ENVIRONMENTAL MICROBIOLOGY AS RELATED TO PLANETARY QUARANTINE

UNIVERSITY OF MINNESOTA

SCHOOL OF PUBLIC HEALTH

ENVIRONMENTAL MICROBIOLOGY
AS RELATED TO PLANETARY QUARANTINE

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PREFACE

This is the report of research activities during the period June 1, 1968, through November 30, 1968, in the project Environmental Microbiology as Related to Planetary Quarantine, an activity of the Environmental Health Division, School of Public Health, University of Minnesota and the National Aeronautics and Space Administration.

This is the first progress report on NASA Grant Number NGL 24-005-160. This is a continuing study; however, for administrative purposes on June 1, 1968, there was a change in project number. The previous work was conducted under NASA Grant Number NGL 24-005-063.

The overall objective of these studies in Environmental Microbiology as Related to Planetary Quarantine is to gather data that will assist in developing specifications to design, build and sterilize planetary exploration hardware which will meet the International Committee on Space Research constraints regarding contamination of the planet.

In this project we have been working in three general areas. These are studies directed toward understanding the behavior of dry heat resistant organisms at ambient conditions, the measurement of dry heat destruction rates at sterilization temperatures and the detection of microorganisms on surfaces using chemical approaches.

To design the final dry heat sterilization cycle for spacecraft hardware it is necessary to know the number of dry heat resistant organisms. Since the required lethality of the sterilization cycle is a function of the number of dry heat resistant microorganisms on the space hardware at the time of sterilization, it would be advantageous to be able to reduce the number of resistant microorganisms. The studies in this project were directed toward developing an understanding of the behavior of dry heat resistant microorganisms at ambient conditions so that we are better able to predict the number of microorganisms on the space hardware at the time of terminal sterilization. Understanding the behavior of these microorganisms may permit us to recommend alterations in spacecraft manufacturing conditions which will reduce the final number of microorganisms on the spacecraft.

The final dry heat terminal sterilization cycle will be based on a dry heat destruction rate. When dry heat sterilization studies were initiated several years ago it was thought that only one destruction or D-value would be required and that this value would be relatively easily obtained since dry heat was thought to be a rather simple destruction process. We now recognize that the dry heat destruction of microorganisms is a very complex process. There are three major variables in dry heat destruction - time, temperature, and the water content of the microorganisms

during the heating period. The water content variable is unique and important since it means that the destruction rate of microorganisms on the spacecraft will be determined by the relative location of these microorganisms. Also, the destruction rate of microorganisms on surfaces and in mated surface areas will be affected by the water content of the gas surrounding the space vehicle during terminal sterilization. During the past six months, we have been actively working to develop dry heat destruction rate values for microorganisms in mated surface areas. These values are badly needed for the 1973 Mars mission. In addition to specific destruction rates we are also interested in developing a better understanding of dry heat destruction rates of microorganisms and specifically the role of water as it affects these destruction rates.

The normal method of assaying for microbial life is a biological culture system. There are many problems associated with assaying surfaces for microbial contamination; the organisms must be mechanically removed through a swabbing or vacuum probe system and then collected and allowed to grow out on solid or in liquid media. It is possible that some of these problems could be eliminated if we were able to analyze for microbial contamination using chemical methods. The studies in this project on chemical methods of determining the number of microorganisms on surfaces are directed toward developing methods which will allow us to assay low

levels of contamination on space hardware using chemical methods.

In this report only the study dealing with survival of Bacillus subtilis var. niger spores in a controlled air stream is new. The study entitled "Survival of Bacillus subtilis var. niger Spores at Temperatures Below 60°C" is a continuation of the study in the report covering the period December 1, 1967, through May 31, 1968, which was entitled "Die-Off of Microbial Contamination". The study entitled "Behavior of Bacillus subtilis var. niger Spores" is, in general, a continuation of work previously reported under the title "Effects of Heat Fixing and Equilibration on Bacillus subtilis var. niger Spores". "Effects of Humidity, Location, Surface Finish, and Separator Thickness on the Heat Destruction of Bacillus subtilis var. niger Spores Located Between Mated Surfaces" is a continuation of the studies entitled "Studies of Attributes of Mated Surfaces That May Affect the Heat Destruction of Microorganisms Located in Those Areas" which was a chapter in the previous report. The study on "Detection of Low Levels of Microbial Contamination on Surfaces by Chemical Approaches" is a continuation of the study which was previously reported under the same title.

During the next six month period all five studies covered in this report will be continued although there may be some small changes in emphasis. It is anticipated that

at least one new study will be initiated during the next six month reporting period. The background of this and any other new study will be reported in our Semiannual Progress Report Number 2.

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SURVIVAL OF *BACILLUS SUBTILIS* VAR. *NIGER* SPORES AT TEMPERATURES BELOW 60°C

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INTRODUCTION

Previous work in this laboratory had been done to evaluate survival of natural bacterial contaminants on stainless steel and epoxy strips under various combinations of temperature and oxygen concentration. These experiments were summarized in the Progress Report covering the period December 1, 1967, through May 31, 1968, at which time work was already underway to evaluate the effect of the air flow in a laminar flow clean room on the survival of *B. subtilis* var. *niger* spores. In the laminar flow clean room studies the survival of *B. subtilis* spores stored in ethanol was compared with spores stored in water. The laminar flow clean room normally operates at a single, constant temperature and humidity. These studies were expanded to several temperature and humidity conditions through the use of small plastic containers in which controlled microenvironments could be developed. One three-week experiment has been concluded and will be reported herein.

OBJECTIVE

The specific objective of these experiments has

been to provide information concerning the survival rates of B. subtilis var. niger spores at temperatures below 60°C over a wide range of relative humidities and also under the influence of a moving airstream.

EXPERIMENTAL PROCEDURE

Experiment 1. A study of the survival of B. subtilis var. niger spores deposited on stainless steel strips and exposed in the airstream of a laminar downflow clean room (90 fpm) maintained at approximately 22°C and 40 percent relative humidity.

In each instance the procedure was as follows:

1. 35 stainless steel strips were contaminated in the manner previously described.
2. 5 strips were randomly selected for analysis at zero time. The remaining 30 strips were placed, contaminated side up, at bench top level in the laminar downflow room.
3. At intervals of 1, 2, 4, 7, 14, and 21 days, five strips were randomly withdrawn for analysis and processed according to NASA Standard Procedures.
4. The experiment was performed once using spores stored in distilled water and once for spores stored in ethanol.

Experiment 2. A study of the survival of B. subtilis var. niger spores over a three week period under various

temperature and humidity combinations.

The procedure was as follows:

1. 296 stainless steel strips were contaminated with 0.1 ml (10^5 spores) from an alcohol suspension of B. subtilis var. niger spores. The deposit was pipetted onto the strip and spread over the surface with a glass rod.
2. The strips were equilibrated overnight in a laminar downflow room (22°C and 40 percent relative humidity).
3. Eight strips were randomly selected for analysis at the end of the equilibration time (0 time). They were analyzed using NASA Standard Procedures for strip analysis.
4. Plastic refrigerator boxes (See Figure 1.1) were set up with silica gel to maintain the following relative humidity conditions:
   a. $<$10 percent, $\sim$50 percent and $>$90 percent at 22°C.
   b. $<$10 percent, $\sim$50 percent and $>$90 percent at 45°C.

   The 22°C condition was obtained by placing an incubator inside a 4°C refrigerator. The 45°C condition was obtained in a high temperature incubator placed in the laboratory

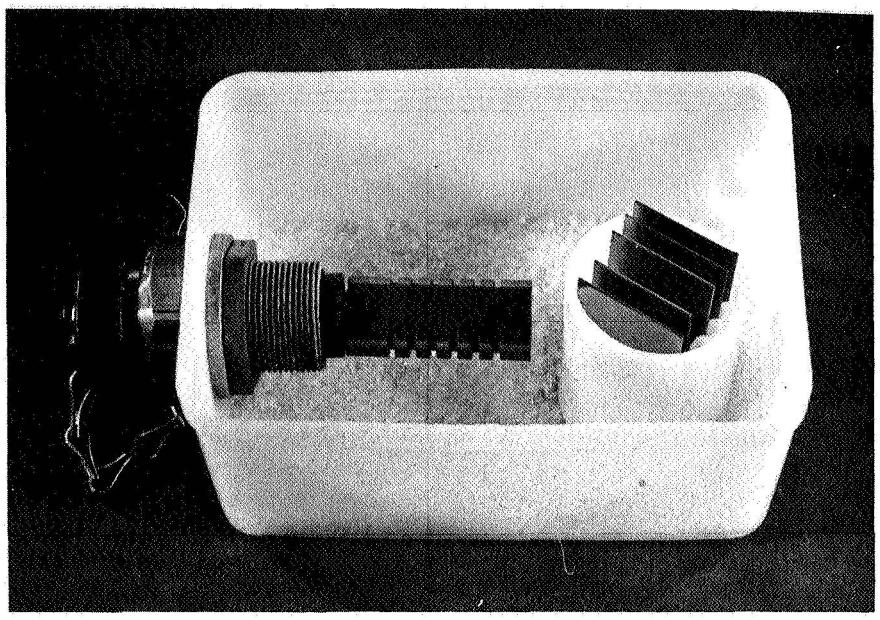

FIGURE 1.1

Plastic container with silica gel, humidity probe and stainless steel strips

5. For each of the six temperature-humidity combinations, six plastic boxes were set up as shown in Figure 1.1. A plastic rack containing eight randomly selected strips was placed in each box. The strips were placed back to back, contaminated side exposed. (In the picture there are three sets of strips back to back and two single strips.)
6. The boxes were sealed, wrapped in flexible plastic film (Parafilm), sealed in plastic bags as shown in Figure 1.2, and placed in the appropriate incubator.

FIGURE 1.2
Plastic container wrapped and sealed

7. Humidity was checked at 3, 9, 16 and 21 days as shown in Figure 1.3. On those occasions the outer plastic bag was opened for the reading, then immediately resealed.
8. For each temperature-humidity combination, one box (8 strips) was withdrawn at 2, 3, 4, 7, 14 and 21 days. The strips were analyzed using NASA Standard Procedures.

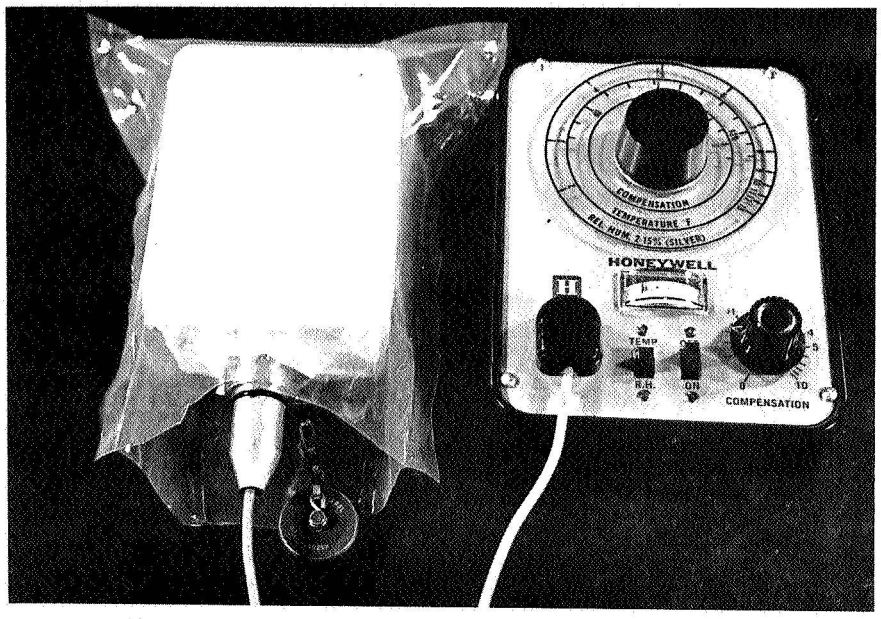

FIGURE 1.3
Relative humidity instrument and microenvironmental chamber

RESULTS AND DISCUSSION

Experiment 1. Table 1.1 summarizes the results for the laminar airflow experiment. It is apparent that the spores from the distilled water suspension died off rather rapidly during the three-week period (from 238 spores/strip to 119 spores/strip). The death rate for the ethanol suspension was much less pronounced.

Experiment 2. Table 1.2 summarizes results of the temperature-humidity experiment. The extremely rapid die-off achieved at 90 percent relative humidity and 45°C was quite surprising. Even at 50 percent relative humidity and

TABLE 1.1

Number of surviving Bacillus subtilis var. niger spores as a function of time and whether the spores were stored in water or ethanol. Spores located on stainless steel strips and exposed in a laminar downflow clean room.

Exposure Time (Days)	Total colonies/strip (mean of 5 strips)	
	Distilled Water Suspension	Ethanol Suspension
0	239.0	672.0
1	237.4	650.4
2	202.4	613.4
4	208.0	680.8
7	182.2	652.2
14	140.0	622.2
21	119.8	566.5

TABLE 1.2

Number of surviving Bacillus subtilis var. niger spores as a function of temperature, humidity and storage time

22°C Temperature

Relative Humidity (percent)	Number of Spores (mean of 8 strips)						
	0 Time	2 Days	3 Days	4 Days	7 Days	14 Days	21 Days
<10	1.7×10^5	1.5×10^5	1.7×10^5	1.6×10^5	1.6×10^5	1.4×10^5	1.6×10^5
50-55	1.7×10^5	1.6×10^5	1.6×10^5	1.2×10^5	1.6×10^5	1.4×10^5	9.2×10^4
>90	1.7×10^5	1.2×10^5	1.1×10^5	1.3×10^5	1.0×10^5	9.2×10^4	2.9×10^4

45°C Temperature

<10	1.7×10^5	1.4×10^5	1.2×10^5	1.3×10^5	1.5×10^5	1.1×10^5	6.0×10^4
50-55	1.7×10^5	1.4×10^5	1.3×10^5	1.1×10^5	7.3×10^4	4.8×10^4	1.3×10^3
>90	1.7×10^5	1.0×10^5	8.8×10^4	4.7×10^4	3.6×10^4	9.5×10^2	$< 10^0$

45°C there was a two log reduction in spore count. At 90 percent relative humidity and 22°C there was almost a one log reduction. Thus it appears that the combination of high humidity and elevated temperature results in rapid die-off of Bacillus subtilis var. niger spores.

CONCLUSION

The results of these experiments suggest that a significant reduction in spore populations can be achieved at temperatures in the range of 45°C with high humidity. However, additional evidence of this phenomena will be sought before attempting to draw definite conclusions.

FUTURE WORK

In the immediate future a longer range (7 month) experiment will be carried out using these same temperature-humidity combinations. Longer range plans include additional similar experiments using distilled water spore suspensions, a different spore species (possibly from the genus Clostridium), and extension of the temperature range to include 60°C.

SURVIVAL OF *BACILLUS SUBTILIS* VAR. *NIGER* SPORES IN A CONTROLLED AIR STREAM

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INTRODUCTION

The survival of the spores of *B. subtilis* var. *niger* has been the focal point of many researchers' work since the conception of NASA's Planetary Quarantine Program. Researchers have studied several parameters that may influence the survival of these spores. The most common parameters evaluated have been time and temperature. D-values have been established for a variety of conditions and many factors have been implicated as the actual cause of death of these cells.

The factor most commonly proposed is that the spore moisture content plays an important role in the death of the cells. Another factor which has been proposed recently is that the velocity of the air across these spores may influence their die-off rate. In a way, this is related to moisture content because velocity should provide the vehicle for moisture removal. Fox (1967) in his work at Michigan State University has indicated that the rate of air flow (at temperatures of 255°F to 285°F) through an enclosed chamber will cause a significant change in the die-off rate. In

this study the air did not flow directly over the surface on which the spores were deposited but passed over the top of small cups. Low flows (1.4 to 4.0 cfm) but high velocities (due to the small area) were used in this study but significant differences were observed indicating a velocity effect.

Vesley and Smith (1968) in their studies on Standard Procedures have shown that microorganisms on inoculated strips placed flat in a vertical downflow air stream (approximately 90 fpm) undergo a die-off rate that is significantly different from the die-off rate of microorganisms on covered strips under the same conditions. Here again, the actual death of the spores may be due to the drying effect of the air stream. Therefore, a study has been undertaken to look deeper into the velocity effect as well as to complement the research already completed in this area.

OBJECTIVE

The objective of this project is to develop D-values based on the three parameters - time, temperature and velocity - for use in the design of NASA spacecraft sterilization procedures and to gain further insight into the effect of air velocity on the survival of B. subtilis var. niger spores.

EXPERIMENTAL PROCEDURE

Equipment

Duct. A plexiglass duct is used to study the effect of the three parameters - time, temperature and velocity. The unit is shown in Figure 2.1. and consists of a 17" long plexiglass duct (1" x 7" internal dimensions) attached to a centrifugal blower. Three recesses were milled in the bottom of the duct with a depth equal to the thickness of standard 1" x 2" stainless steel strips. The recesses make the top of the strips flush with the bottom of the duct, therefore subjecting the deposited spores to a direct air stream.

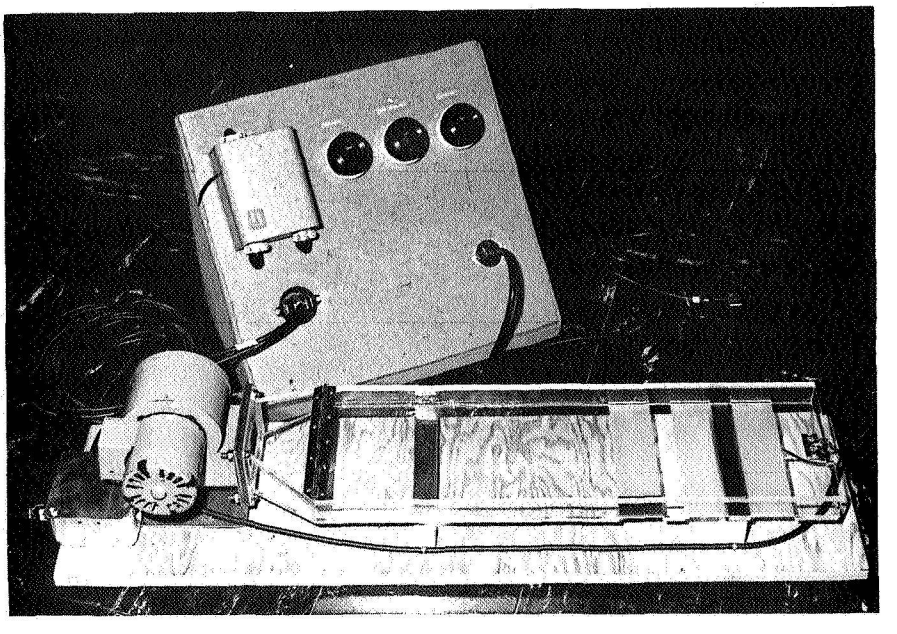

FIGURE 2.1
Control panel and plexiglass duct

The top of the duct was hinged for easy access to the strips. An electrical resistance heater was placed in the inlet of the blower. A thermistor was placed in the end of the duct and connected to a transistorized amplifier relay to control the heater and regulate the temperature of the air.

Strips. Stainless steel strips were used as the surface for deposition of the B. subtilis var. niger spores. The strips are 1" x 2" and .024" thick with a brushed finish. The spores were deposited on the strips and recovered using standard NASA laboratory procedures.

Procedure

The unit was designed to hold 18 strips. A total of 42 strips are prepared for each run. Thirty-six of the contaminated strips are randomly divided into two groups of eighteen each; the remaining six strips serve as an initial count control.

One group of 18 strips is divided into three subgroups of 6 strips each and serves as controls for each time interval. The other group of 18 strips is placed in the slots of the duct, six strips per row.

The air flow and air temperature control systems are adjusted until the desired velocity and temperature are attained. The strips are then loaded into the system and the test carried on for the initial time interval. After

the initial time interval, six strips are removed from the duct and the viable spores recovered. Six controls are also removed at this time. The six empty positions in the duct are filled with sterile strips and the run is continued until the next time interval has elapsed. The recovery technique is repeated until the run is completed.

RESULTS AND DISCUSSION

The strips were removed from the duct in a manner that allowed a statistical examination of the position effect as well as the effect of time and temperature. A Latin Square technique was employed to examine the position effect. This technique requires the data in row-column format with equal numbers of each. Since two strips were removed at each row-column position for each of the time intervals, duplicate Latin Squares were required. Latin Squares were constructed and the data were examined for row, column and time effect.

The strips were arranged in the duct as shown in Figure 2.2. A, B and C represent the three time intervals of one, two and three weeks.

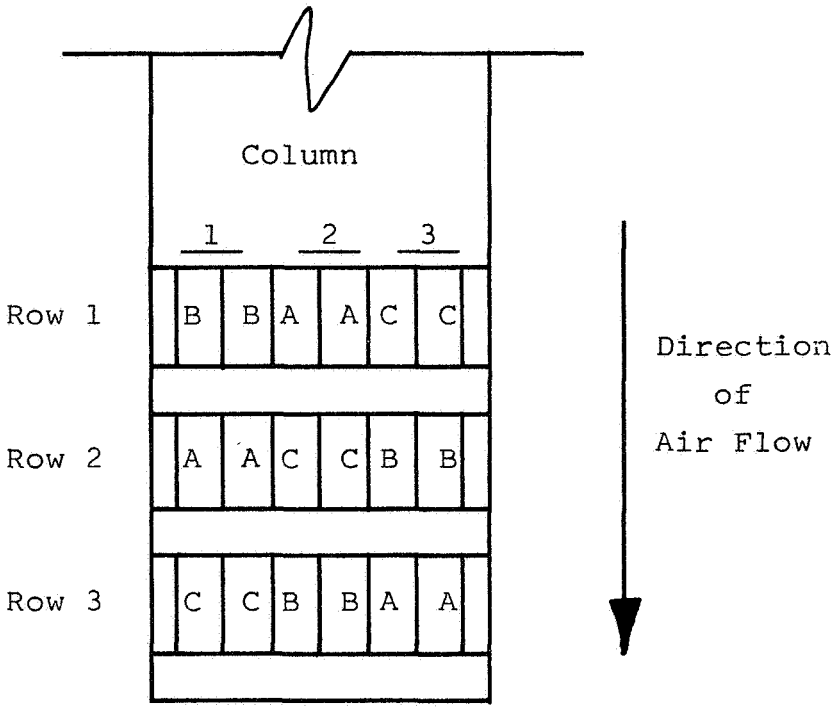

FIGURE 2.2
Layout of exposed strips

Tables 2.1, 2.2 and 2.3 display the data obtained from three runs. All the runs were made in vertical laminar flow clean room at ambient room conditions. The counts shown in the tables are the average of duplicate plate counts obtained from the recovery process. To obtain the total number of organisms surviving on the strips the plate counts are multiplied by 500.

TABLE 2.1

Summary of Data

Run number 1
Velocity 745 fpm

Temp 25°C
R.H. 45%

Latin Square I

Row \ Column	1	2	3	Sum
1	B 205	A 249	C 265	719
2	A 226	C 212	B 228	666
3	C 167	B 217	A 203	587
Sum	598	678	696	

Sum	A	B	C
	678	650	644

Latin Square II

Row \ Column	1	2	3	Sum
1	B 235	A 149	C 252	636
2	A 231	C 187	B 223	641
3	C 163	B 183	A 233	579
Sum	629	519	708	

Sum	A	B	C
	613	641	602

Analysis of Variance

Source of Variation	df	S.S.		M.S.		F	
		I	II	I	II	I	II
Rows	2	2941	791	1470	395	2.13	.22
Columns	2	1814	6007	907	3004	1.31	1.71
Time	2	219	270	110	135	1.6	.07
Error	2	1381	3520	691	1760		
Total	8	6355	10588				

Average and Coefficient of Variation of Counts

Week	Controls		Exposed	
	Avg	CoV	Avg	CoV
Initial	---	---	---	---
A	229	8.2%	215	16.5%
B	212	13.7%	215	8.6%
C	243	14.1%	208	20.7%
Total	228	13.2%	121	15.2%

TABLE 2.2

Summary of Data

Run number 2
Velocity 1450 fpm

Temp 25°C
R.H. 45%

Latin Square I

Row \ Column	1	2	3	Sum
1	B 215	A 249	C 240	704
2	A 221	C 293	B 271	785
3	C 234	B 255	A 250	739
Sum	670	797	761	

Sum	A	B	C
	720	741	767

Latin Square II

Row \ Column	1	2	3	Sum
1	B 241	A 255	C 234	730
2	A 217	C 256	B 224	697
3	C 238	B 314	A 247	799
Sum	696	825	705	

Sum	A	B	C
	719	779	728

Analysis of Variance

Source of Variation	df	S.S.		M.S.		F	
		I	II	I	II	I	II
Rows	2	1100	1806	550	903	3.07	4.68
Columns	2	2856	3458	1428	1729	7.98	8.96
Time	2	369	698	185	349	1.03	1.80
Error	2	359	386	179	193		
Total	8	4684	6348				

Average and Coefficient of Variation of Counts

Week	Controls		Exposed	
	Avg	CoV	Avg	CoV
Initial	277	10.9%	---	---
A	245	7.2%	239	5.4%
B	257	8.9%	253	14.2%
C	252	9.9%	249	9.2%
Total	251	6.4%	247	10.3%

TABLE 2.3

Summary of Data

Run number 3
Velocity 220 fpm

Temp 25°C
R.H. 45%

Latin Square I

Row \ Column	1	2	3	Sum
1	B 251	A 273	C 254	778
2	A 276	C 250	B 236	762
3	C 268	B 226	A 280	774
Sum	795	749	770	

Sum	A	B	C
	829	713	772

Latin Square II

Row \ Column	1	2	3	Sum
1	B 257	A 278	C 248	783
2	A 270	C 266	B 255	791
3	C 258	B 244	A 278	780
Sum	785	788	781	

Sum	A	B	C
	826	756	772

Analysis of Variance

Source of Variation	df	S.S.		M.S.		F	
		I	II	I	II	I	II
Rows	2	46	21	23	10	.37	.07
Columns	2	353	8	176	4	2.88	0.0
Time	2	2243	896	1121	448	18.3	3.27
Error	2	121	275	61	137		
Total	8	2763	1200				

Average and Coefficient of Variation of Counts

Week	Controls		Exposed	
	Avg	CoV	Avg	CoV
Initial	254	7.7%	---	---
A	255	4.2%	275	1.4%
B	253	4.5%	245	4.9%
C	271	4.5%	257	3.1%
Total	259	5.3%	259	5.9%

The results from these preliminary runs are very interesting. In the Analysis of Variance Table where the position effect was analyzed, an F test was performed. A significant F at the 5% level for 2 x 2 degrees of freedom is 19.00. The F-test did not exceed this value in any run. The test was not even close to significant except for the time effect in Run #3. The F-test for this run yielded an F of 18.3. The air velocity in Run #3 was the lowest of any of the runs (220 fpm). This result seems to indicate that there might be a significant die-off at a lower velocity. If this condition does exist, it would be in support of the previously mentioned study of Vesley and Smith.

When all the control strips and exposed strips are averaged and compared for each of the runs, the difference in means is not statistically significant. However, in all except the third run, the average value for the exposed strips is lower than the control strips which is what might be expected and may indicate a trend.

FUTURE WORK

In the next reporting period runs will be completed with approximately the same velocities but at an elevated temperature. The temperature will be in the range of 40-45°C. When these runs are completed it will be possible to determine more explicitly the effect of time, temperature and velocity and also the interaction of these three factors.

BEHAVIOR OF *BACILLUS SUBTILIS* VAR. *NIGER* SPORES

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Division of Environmental Health

INTRODUCTION

The diversified results of different laboratories have indicated the need for evaluation of factors affecting the recovery and D-values of *Bacillus subtilis* var. *niger* spores. Previous experiments on the effect of heat fixing spores at 80°C and their subsequent recovery were reported in the Progress Report covering the period December 1, 1967, through May 31, 1968. Reported herein are the concluding experiments on heat fixing and an initial experiment designed to develop a method for standardizing the D-value of spore crops.

OBJECTIVE

Experiment 1. The objective of this study is to determine the effect of heat fix time (at 80°C) on recovery of *B. subtilis* var. *niger* spores suspended in ethanol and distilled water.

Experiment 2. The objective of this experiment is to determine the effect of different quantities of moisture on $D_{125°C}$-values obtained on *B. subtilis* var. *niger* spores in thermal death time (TDT) cans.

EXPERIMENTAL PROCEDURE

Experiment 1. Two identical sub-experiments were performed; however, in the first test the spores were suspended in distilled water and in the second they were suspended in 95 percent ethanol. In each case 40 strips were contaminated, then randomly allocated to the following treatments.

1. 5 strips not heated, control
2. 5 strips heated 20 minutes dry
3. 5 strips heated 60 minutes dry
4. 5 strips heated 20 minutes wet
5. 5 strips heated 60 minutes wet
6. 5 strips conditioned 24 hours in laminar flow room (22°C, 40 percent relative humidity) not heated
7. 5 strips conditioned 24 hours in laminar flow room heated 20 minutes
8. 5 strips conditioned 24 hours in laminar flow room heated 60 minutes

Strips were processed according to NASA Standard Procedures as previously reported.

Experiment 2. Sealed TDT cans, each containing four cups, were prepared as follows.

1. Approximately 10^5 spores were added to each cup from a distilled water suspension.

2. Cups were equilibrated overnight in a laminar downflow room at 22°C and 40 percent relative humidity.
3. Water was added to small pieces of blotting paper (also equilibrated at 22°C and 40% RH) in the TDT cans. The paper was folded so that the moistened portion did not contact the cups.
4. Four cups were placed in each can and two cans (8 cups) were processed for each moisture condition.
5. The cans were sealed immediately after being prepared and heated in an oil bath at 125°C for 40 minutes.
6. After heating the cans were cooled by placing them into cold water.
7. Processing was carried out the following day using NASA Standard Procedures. Duplicate dilutions were made for each cup.
8. The conditions evaluated were as follows:
   a. unheated control - no moisture, no blotting paper
   b. heated 125°C - 40 minutes - no moisture, no blotting paper
   c. heated 125°C - 40 minutes, no moisture, with blotting paper

d. heated 125°C - 40 minutes - 0.001 ml H_2O, with blotting paper

e. heated 125°C - 40 minutes - 0.01 ml H_2O, with blotting paper

f. heated 125°C - 40 minutes - 0.02 ml H_2O, with blotting paper

RESULTS AND DISCUSSION

Experiment 1. The results are reported in Table 3.1. They confirm that heating at 80°C does not significantly affect spore recovery. In all cases, except the unconditioned ethanol control, the percent removal by sonication was extremely high (99.5 to 100 percent) and did not appear to be affected by the heating time. All counts of water suspended spores were lower after heating and 24 hour conditioning. Although the difference is small, these results confirm the survival experiment "Survival of *Bacillus subtilis* var. *niger* Spores at Temperatures Below 60°C, Experiment 1," contained earlier in this report. Colony counts of ethanol suspended spores were not affected by either heating or conditioning time.

Experiment 2. Results are summarized in Table 3.2. It is apparent that water content in the sealed cans had a significant effect on the destruction rate. The addition of 0.01 ml or 0.02 ml sharply reduced the D-value compared to those values achieved with less than 0.001 ml of H_2O.

TABLE 3.1

Comparison of 20 minute versus 60-minute 80°C heat fix in recovery of distilled water and ethanol suspensions of *Bacillus subtilis* var. *niger* spores from stainless steel strips

Suspension	Strip condition	Heat fix time					
		Unheated controls		20 minutes		60 minutes	
		Total col/ strip*	% removal	Total col/ strip*	% removal	Total col/ strip*	% removal
Distilled Water	Wet	-	-	142.4	100.0	133.4	100.0
	Dry	149.4	100.0	149.3	100.0	145.6	99.5
	Conditioned 24 hours LFR	141.0	100.0	109.8	99.6	102.6	100.0
	Overall time effect	145.2	100.0	133.8	99.9	127.2	99.8
Ethanol	Wet	-	-	509.2	99.7	524.8	100.0
	Dry	501.0	93.3	534.0	100.0	496.0	99.6
	Conditioned 24 hours LFR	570.6	99.7	523.4	100.0	534.6	100.0
	Overall time effect	535.8	99.5	522.2	99.9	518.5	99.9

* - Based on 5 strips/condition
LFR - Laminar flow room

TABLE 3.2

$D_{125°C}$-values for Bacillus subtilis var. niger spores heated under varying moisture conditions

Condition	Mean survivors/cup*	$D_{125°C}$-value (minutes)**
Unheated controls (no H_2O or blotting paper)	9.4×10^4	-
No H_2O or blotting paper	2.6×10^3	25
No H_2O with blotting paper	2.5×10^4	70
0.001 ml H_2O, with blotting paper	2.5×10^4	70
0.01 ml H_2O, with blotting paper	9.7×10^1	13
0.02 ml H_2O, with blotting paper	3.8×10^0	8.5

* - Based on 8 cups/condition

** - Based on mean for unheated controls

The higher D-values reported for the small quantities of water, 70 minutes for 0.001 ml water and for the equilibrated blotting paper with no water added, compared to the control, with no water or blotting paper, again indicates that there is a critical small quantity of water which affords some protection to the spores. (In this single test we do not know if 70 minutes is the the maximum $D_{125°C}$-value.) This is a preliminary test to a more complete study of the effect of water in the system.

CONCLUSIONS

Experiment 1. Heating time (up to one hour at 80°C) did not appear to affect recovery of B. subtilis var. niger spores. The survival of water suspended spores was affected by heating and conditioning times. It would seem that the removal of water from the spore is very important to its survival.

Experiment 2. It is possible to manipulate D-values of B. subtilis var. niger spores in thermal death time cans by adding different quantities of water to the can.

FUTURE WORK

Work is underway in this laboratory to standardize as much as possible the methodology for obtaining dry heat D-values.

EFFECTS OF HUMIDITY, LOCATION, SURFACE FINISH, AND SEPARATOR THICKNESS ON THE HEAT DESTRUCTION OF *BACILLUS SUBTILIS* VAR. *NIGER* SPORES LOCATED BETWEEN MATED SURFACES

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INTRODUCTION

The project is attempting to relate the thermal destruction of bacterial spores between mated surfaces to certain parameters of the environment and the surfaces.

In previous reporting periods the experimental apparatus was designed and constructed, experimental procedures and plans were prepared and two initial test runs were carried out to test procedures and to assist in planning.

The current reporting period was largely devoted to the collection of experimental data to help determine which parameters will be examined in detail and to the measurement of characteristics of the experimental system which may affect the results.

OBJECTIVE

The project will measure the thermal death parameters of *B. subtilis* var. *niger* spores in the mated surface system and will measure the effect of certain variations of the environment and of the surfaces on thermal death.

EXPERIMENTAL PROCEDURE

The experimental apparatus is shown in Figure 4.1; it consists of two 10 x 10 x 2 inch aluminum blocks held together with springs. Each block is heated by four cartridge heaters; the blocks are independently temperature controlled to a maximum accuracy of $\pm 1°C$ by Honeywell thermistor controllers. Power input is controlled by a variable autotransformer. Two sheets of stainless steel shim stock (.015" thickness) are the mated surface under test. In early experimental runs the surfaces were wrapped in aluminum foil to exclude contamination. In later runs a mylar-polypropylene heat sealed package is used; the latter is easier to handle and appears to provide a tighter seal on the

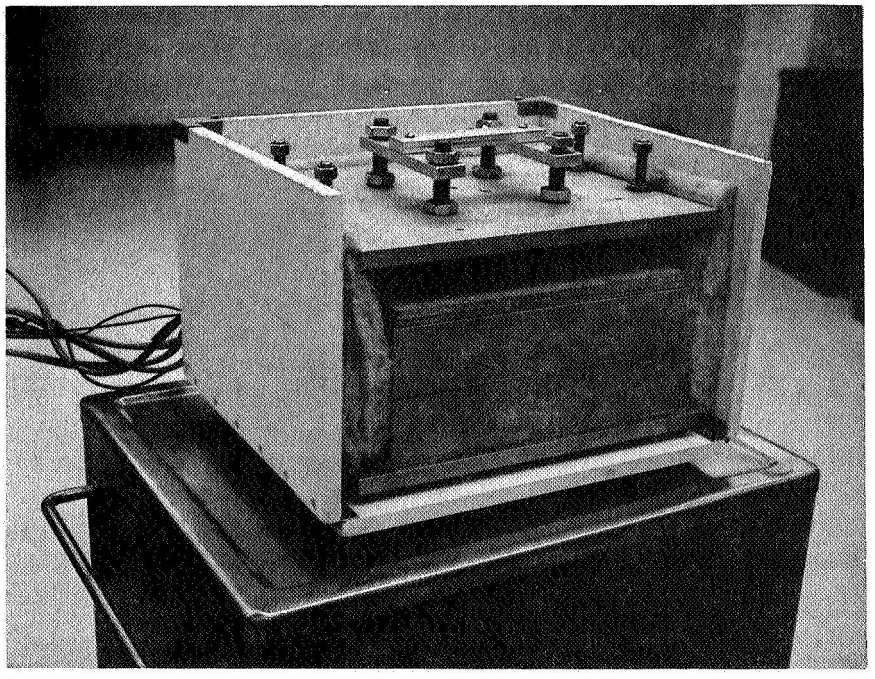

FIGURE 4.1

The heat block showing insulation which was added to improve temperature uniformity; additional heaters have also been added.

system. Figure 4.2 shows the packaged and unpackaged mated surfaces along with a separator which is optional; the marks on the exposed surface represent the locations of the spore deposits.

The following description of procedures applies in broad detail to all runs after Number 3 except as noted in the descriptions of the individual runs; in finer detail it is the current procedure for carrying out the experimental runs.

The spore suspension of proper dilution is placed in a convenient container. A 20 microliter Eppendorf micropipette is used to deposit approximately 1.3×10^5 spores on the base plates. Tips for the Eppendorf micropipette are

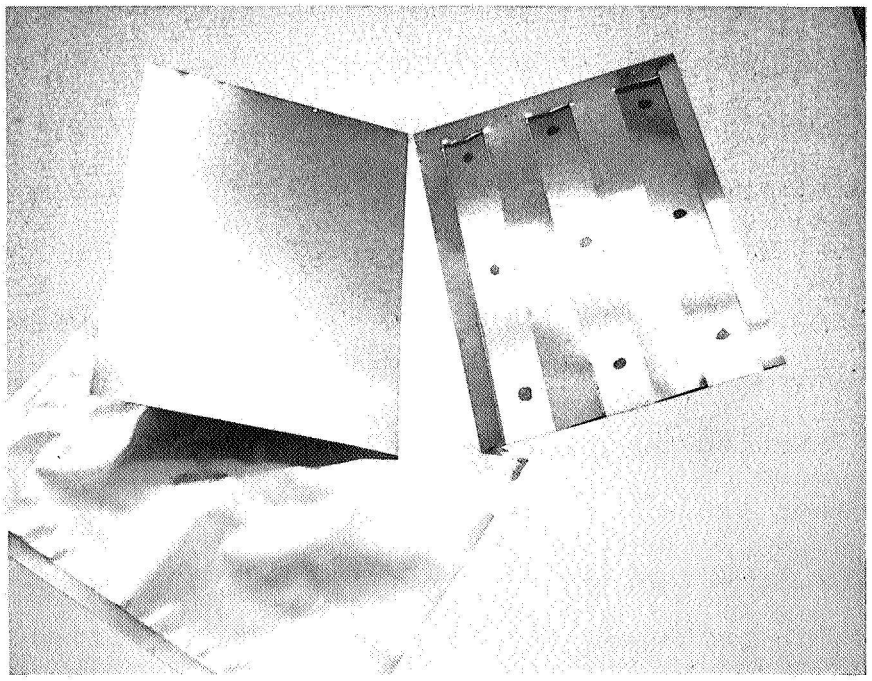

FIGURE 4.2

The mated surface package open to show the location of spore deposits.

individually wrapped and sterilized with ethylene oxide more than three days prior to use. The spore suspension is agitated at least after completion of each plate, that is after each nine deposits. A time separation of about two to three minutes between agitations is involved.

The base plates are individually wrapped and sterilized with ethylene oxide. Before use they are unwrapped and laid in sterile shallow trays in the laminar downflow clean room. After the spores are deposited, the trays are covered loosely and the base plates are allowed to equilibrate overnight with the ambient atmosphere in the clean room, about 22°C and 30% RH. In the morning the cover plates, which are also individually wrapped and ethylene oxide sterilized, are placed on top of the base plates and, handling the mated surface unit with sterile gloves, it is slipped into a mylar-polypropylene package which is presealed except at one end. The package has been presterilized in ethylene oxide. The top and front of the package are labeled for proper orientation in the heat block; the packages are then carried to the work area in the sub-basement where they are heat sealed. Prior to sealing, the package is placed inside a book which is held shut to exclude most of the air from the package. The package is then carried to the location of the heat block for treatment.

For processing, the package is returned to the

laminar downflow clean room. Handling it with sterile gloves, the package is opened and the mated surface unit is removed. Each spore deposit is cut from the baseplate on a 1 x 2 inch strip with sterile tinsnips or a sterile bench shear and is placed in a 125 ml Erlenmeyer flask. It is generally left there overnight before further processing.

The strips are processed consistent with the NASA Standard Procedures. Fifty ml of sterile buffer is added to the flask containing the strip; the flask is sonicated for two minutes at the center of a full ultrasonic bath filled with sterile .3% Tween 80 solution. 0.1, 1.0 or 10.0 ml aliquots are plated in duplicate. Trypticase soy agar is used in all experiments; the agar is contained in individual tubes. The water bath used to hold the tubes of agar is filled with a solution of Halimide to prevent contamination. Accidental contamination of a plate by a drop from the water bath will result in a zero rather than a high count. Plates are incubated for 48 hours before being counted.

A computer data handling system had been developed to assist the project; however, after revisions in the experimental procedures, the data handling system has become obsolescent. Therefore, data processing to date has been performed manually using a desk calculator. Rather than extensively revising the former computer system, it will be abandoned and the statistical services group of the Biometry

Department will be commissioned to design a new system.

To measure heat-up and cool-down times of the mated surface package a dummy package containing thermocouples located in the positions of the spore deposits has been constructed. The heat capacity and thermal heat conductivity of the dummy system are matched as closely as possible to the real test package.

The dummy package will be used to measure the exact temperature at the treatment locations of the heat block as a function of the monitored temperature and to measure the heat-up and cool-down of the treatment packages.

Some variation in the temperature from point to point in the heat block is inevitable, as is a variation in temperature from run to run. To remove variations in the data from this source, a mathematical correction can be applied.

The equation of the survivor curve is:

(1) $$\log N_{t_T} = -\frac{\Delta t}{D_T} + \log N_0$$

where:

N_{t_T} = number of organisms surviving at time t after treatment at temperature T

N_0 = starting number of organisms (number at t = 0)

Δt = elapsed treatment time = $t - t_0$

D_T = decimal reduction time (D-value) at temperature T (°C)

The relation between D, temperature and z is similar

(2) $$\log D_T = -\frac{\Delta T}{z} + \log D_0$$

where:

D_0 = D-value at temperature T_0

D_T = D-value at temperature $T + \Delta T$

$\Delta T = T - T_0$

T = treatment temperature (°C)

z = temperature change to give a one log change in D

In the present experiment:

z = 21°C

T_0 = 125°C. $D_0 = D_{125}$

N_{t_T} is estimated from the number of organisms recovered after treatment.

N_0 is estimated from the average recovery from the wrapped control.

T and t are measured.

We want to estimate $N_{t_{125}}$, the number of organisms which would have survived if the treatment temperature had been 125°C.

We can write

$$\log N_{t_{125}} = -\frac{\Delta t}{D_{125}} + \log N_0$$

Rearranging (2):

$$\log D_{125} = \log D_T + \frac{T - 125}{z} = \log D_T + \frac{T - 125}{21}$$

Combining the two equations gives

$$(3) \qquad \log N_{t_{125}} = \frac{-\Delta t}{\text{antilog } (\log D_T + \frac{T - 125}{21})} + \log N_0$$

Finally D_T is obtained by rearranging (1). It is an estimate based on the slope of the line connecting N_0 and N_{t_T} on a semi-log plot.

$$(4) \qquad D_T = - \frac{\Delta t}{\log N_{t_T} - \log N_0} = - \frac{\Delta t}{\log \left(\frac{N_{t_T}}{N_0} \right)}$$

The estimate of D_T, which may also be obtained from a graph, is placed in (3) which can then be solved for $N_{t_{125}}$.

RESULTS AND DISCUSSION

The experimental runs of the present reporting period have primarily been to explore the scope of the problem of survival in mated surfaces. Since there have been few replicate experiments, only a limited amount of statistical analysis has been possible and the results which are reported should be considered as preliminary data. Individual runs are reported in summary form near the end of this section. The semi-raw data of a few typical runs follow the report.

As a partial check on the experimental procedure the wrapped controls of Runs 4-8 were analyzed to determine

whether the variations in recovered numbers are truly random. An analysis of variance using a factorial design with replication and sampling was performed on the raw plate counts. The results are as follows:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square
Replicates (runs)	4	10619	2655
A (rows)	2	1631	815
B (columns)	2	4318	2159
A x B (interaction)	4	3364	841
Error (within Sampling spore deposit)	32	36776	1150
	45	14337	319
Total	89	71045	

None of the variations are significant at the 5% level.

An unexpected discovery was the large effect on D-value of a small change in the dew point of the treatment environment. The effect was first noticed quite accidentally; after the first several runs had yielded a D-value in the neighborhood of 60 to 65 minutes, the D-value suddenly dropped to 30 minutes and then to 25 minutes. In retrospect, the change was associated with the change from humid summer weather to cool fall weather and with the move to an air conditioned laboratory. The association was confirmed by conducting an experimental run (#6) in a humidified environment.

It should be noted that except in Run #9 the numbers

of recovered spores are not corrected for variations in the monitored temperature of the heat block. In no case are the counts corrected for temperature variations between the monitored temperature and the temperature at the spore exposure position. This data is available and will be used in the future to correct the results of the past runs. The corrections will be minor and should have little effect on any conclusions reached.

The effect of heat-up and cool-down on the data has not been evaluated. The heat-up and cool-down characteristics measured in the dummy package are shown in Figure 4.3.

A strong association has also been noted between

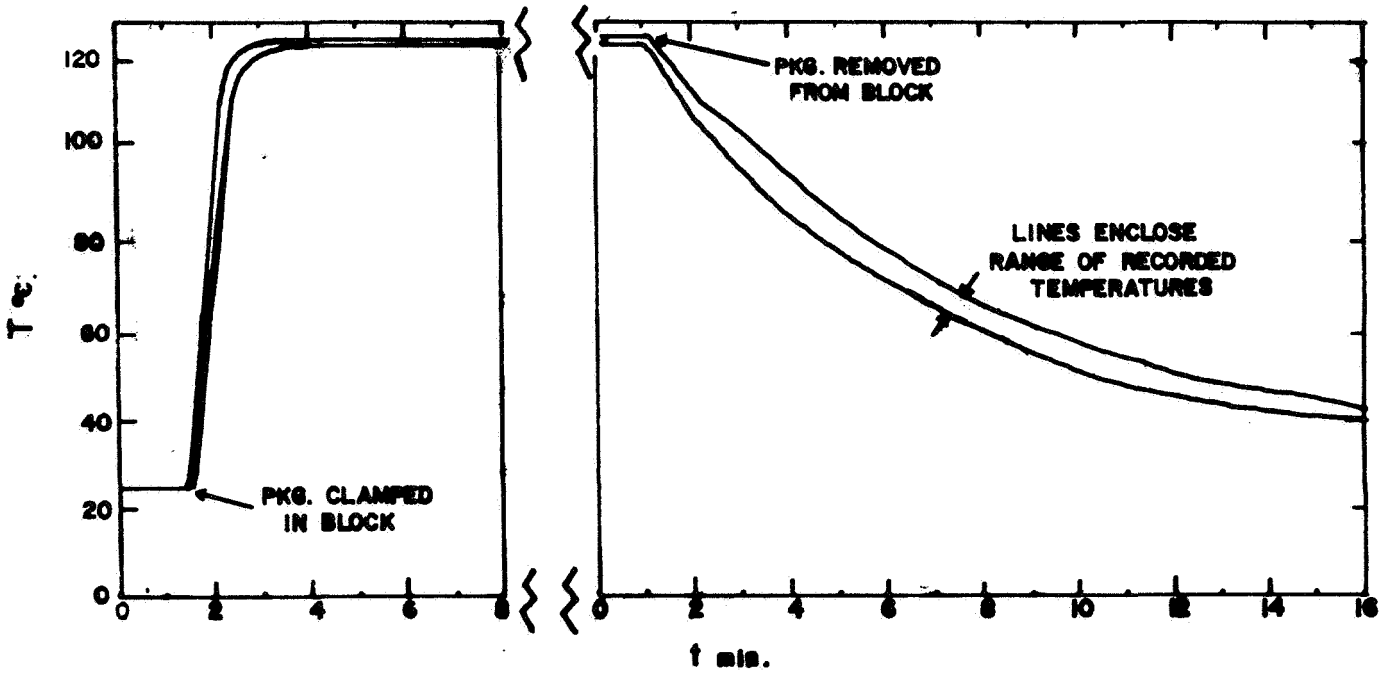

FIGURE 4.3

Heat-up and cool-down characteristics of the mated surface package, measured with thermocouples at the location of the spore deposits

surface finish and D-value as shown in the results of Run #13.

SUMMARIES OF EXPERIMENTAL RUNS

Run #1, June 25, 1968

The run used type 302 stainless steel without separators. There were 20 spore deposits per plate.

Packages were foil wrapped.

D = 66 min.

Run #2, July 18, 1968

A standard base plate and layout for spore deposits on the base plate have been adopted as shown in Figure 4.4. The use of nine spore deposits per plate is a compromise between the need to examine various locations in the mated surface area and the need to include a number of conditions in a given experiment. Run #2 included a sequence of standard packages plus two modified packages which were exposed for 80 minutes:

1. A package with spores deposited ½ inch from the edge of the base plate as shown in Figure 4.5.
2. A standard package whose exposed front end was not cut off during treatment.

The differences between the three packages were not significant.

To decrease temperature overshoot in the heat block, a variable transformer was inserted in the heater line for this and all subsequent runs. The temperature fluctuation was decreased from ±3°F in Run #1 to ±2°F in the present run. Packages were foil wrapped.

D = 55 min.

Run #3, August 8, 1968

The following packages were included:

1. Standard foil wrapped packages exposed at 20 and 80 min.
2. A package with a .0005 inch separator exposed for 80 min.

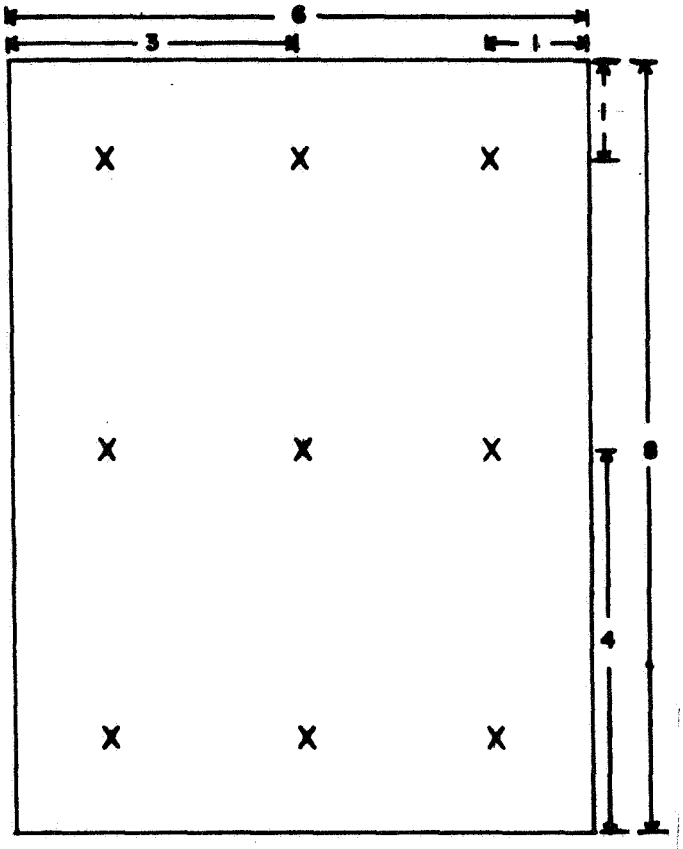

FIGURE 4.4

Standard layout
of spore deposits

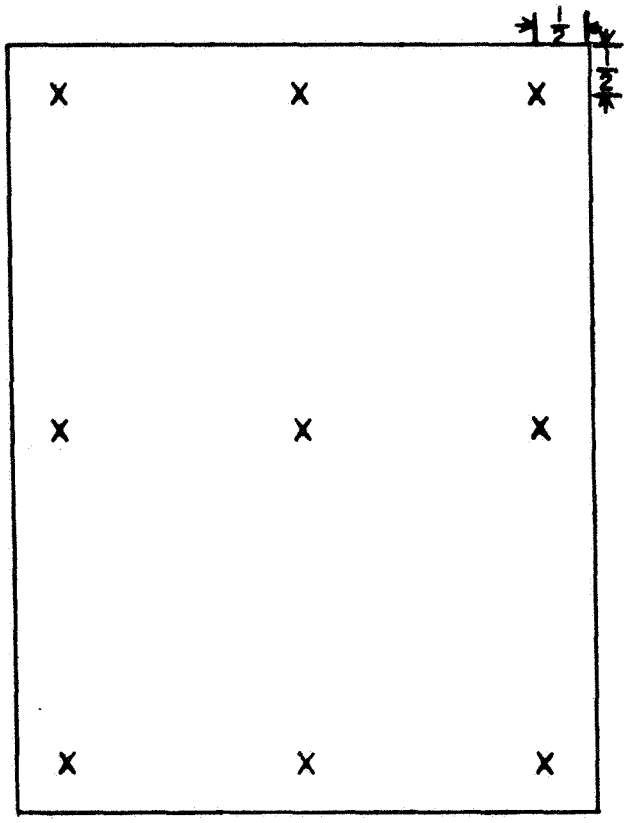

FIGURE 4.5

Modified layout
of spore deposits

3. A package with a .005 inch separator exposed for 80 min.
4. A package in a mylar-polypropylene heat sealed envelope from which the end was not cut off, exposed for 80 min.

There is a great deal of variability in this data, probably due to defective operation of the Misco lambda pipetter used to deposit the spore suspension. In future runs an Eppendorf micropipette will be used.

A second source of variation in the data is evidenced by a temperature map of the heat block which shows a variation of about $\pm 4°F$ including a left to right variation. To increase uniformity and to eliminate the left to right variation, four cartridge heaters per block will be installed instead of two. The heaters will be operated at reduced power so that fluctuation due to overshoot will also be decreased.

The mylar package shows a higher survival after treatment indicating that it is less permeable than the foil wrap as would be

expected. It was intended that the mylar package be completely sealed; however, air trapped in the package created a small gap in the heat seal when the package was clamped between the blocks. The average D-value of the packages with separators is somewhat lower than that of the regular package as would also be expected due to the openings produced by the separators.

$D_{\text{std. pkg.}}$ = 66 min.

$D_{\text{mylar pkg.}}$ = 88 min.

$D_{\text{std. pkg. with separator}}$ = 48 min.

Run #4, August 29, 1968

Mated surfaces of type 301 stainless steel were used; they have a slightly more polished surface than the type 302 plates used in Runs #1-3. Type 301 has since been shown to give a longer survival than type 302.

The run investigated the variable of plate separator thickness at a treatment time of 80 minutes; separator thickness varied between zero and .015 inches. The D-values showed a decrease with increasing separator thickness as would be expected.

The run was performed after a change from humid summer weather to cool fall weather in the non-air-conditioned laboratory. Although the dew point was not measured during the run, the decrease in D-value from previous runs is now attributed to a decrease in dew point.

$D_{\text{std. pkg.}}$ = 32 min.

$D_{.0005\text{" sep.}}$ = 28 min.

$D_{.005\text{" sep.}}$ = 28 min.

$D_{.015\text{" sep.}}$ = 24 min.

Run #5, September 12, 1968

Two types of foil wrapped packages were used in the test. They are referred to as regular and wet. To prepare the wet packages, spores were deposited on the base plate in the normal manner but as soon as the visible water droplet evaporated, the package was

wrapped and put under a light weight overnight. To prepare the regular package, the base plate is put in a loosely covered tray and allowed to equilibrate overnight with the clean room atmosphere before being wrapped. The allocation of packages to treatments is as follows:

	Regular	Wet
Wrapped control	X	X
20 minutes	X	-
45 minutes	X	X
80 minutes	X	-
120 minutes	X	X

In an attempt to reduce the variability of the data the spore suspension was sonicated for three minutes before deposition on the base plates. No change in variability was noted.

No consistent difference between regular and wet packages was visible; the foil package may be sufficiently permeable that water vapor is lost in spite of being wrapped and weighted.

D = 23 min.

Run #6, September 25, 1968

The run was performed to confirm the suspicion that the shorter D-values of Runs #4 and 5 were caused by a lower dew point during treatment of the spores. The plates were treated in a humidified incubator which had a dry bulb temperature of 90°F and a wet bulb temperature of 69°F. Packages and treatments were as follows:

	Regular	Wet
Wrapped control	X	X
20 minutes	X	-
40 minutes	X	X
80 minutes	X	-
160 minutes	X	X

The wet packages were prepared as for Run #5; again there were no consistent differences between the regular and wet packages. As in Run #5 the packages were foil wrapped and type 301 stainless steel was used for the

mated surface.

$D = 50$ min.

Run #7, September 30, 1968

The run was performed to compare the foil package used in early runs with the mylar package tested briefly in Run #3. Both packages were exposed at 20, 50 and 100 minutes and were prepared as wrapped controls. The treatment environment had a dry bulb temperature of 76°F and a wet bulb temperature of 63°F to give a relative humidity of about 49%.

At treatment times of 20 and 50 minutes there was no detectable difference between the foil and the mylar-polypropylene packages. At the 100 minute treatment the foil packages showed a considerable lower survival than did the mylar package. The explanation for the sudden divergence is unknown.

$D_{\text{foil pkg.}} = 42$ min.

$D_{\text{mylar pkg.}} = 58$ min.

NOTE: Henceforth, the "Standard Package" is mylar-polypropylene wrapped.

Run #8, October 3, 1968

The standard mylar package with the end cut off was compared to the behavior of a package left sealed during treatment. Type 301 stainless steel was used for the comparison. In addition, a standard package using type 302 stainless steel was exposed for 110 minutes to begin to measure the difference between the two types of stainless steel.

	Sealed Package Type 301	Open End Package Type 301	Open End Package Type 302
Wrapped control	X	-	-
20 minutes	X	X	-
50 minutes	X	X	-
110 minutes	X	X	X

The treatment environment had a dry bulb temperature of 75°F and a wet bulb temperature of 58°F.

There was no measurable difference

between the means of the sealed and unsealed packages except at 110 minutes; even there the difference was not statistically significant. There was, however, a consistent difference in the variation within packages with the unsealed (regular) package having about one-half the standard deviation of the sealed package. It is due to a more rapid destruction in the front row of the unsealed package due to more rapid diffusion of water from the front row into the relatively close external environment.

The type 302 package showed a slightly lower mean than the type 301 packages and it showed a very large front to rear difference within the package, almost two orders of magnitude in row means. The type 302 stainless steel has a somewhat rougher surface than the type 301 and has brush marks which run from the front to the rear of the package; both facts should facilitate diffusion.

$D_{301\ \text{unsealed}} = 50$ min.

$D_{301\ \text{sealed}} = 46$ min.

$D_{302\ \text{unsealed}} = 40$ min.

Run #9, October 14, 1968

The run was carried out with the heat block in a refrigerator to measure the effect of a reduced dew point on D-value. The dry bulb temperature of the refrigerator was 48°F; the wet bulb temperature was 42.5°F. The run used type 301 stainless steel plates treated at 20, 40, 80, and 120 minutes and additionally a type 302 stainless steel plate exposed at 80 minutes. Temperatures in the heat block were not completely constant during the run, ranging from 110°C during the 20 minute treatment up to 125°C during the 120 minute treatment. To measure the D-value of the spores at 125°C, the counts have been corrected using a z-value of 21°C according to the procedure described in the Experimental Procedure section.

The results show no difference between type 301 and type 302 stainless steel in this run. This is different from the result obtained earlier; the discrepancy cannot be explained at this time.

D = 30 min.

Run #10, October 17, 1968

The run was an attempt to compare the spores which have been used in previous runs with a new crop of spores produced recently in our own laboratories. The spores used in runs 1 through 9 had been stored in alcohol suspension in a freezer for about one year and had been spun down, resuspended in water, divided into aliquots and refrozen. The new crop of spores has seen only a water environment and has not been frozen. The temperatures of the treatment environment were dry bulb 75°F, wet bulb 61°F. D-values in the neighborhood of 15 minutes were obtained for both crops of spores although the data is far too inaccurate to allow a closer estimate. We can say quite definitely, however, that the older spores with a history of alcohol storage are more resistant to dry heat than is the new crop of spores.

Run #11, October 21, 1968

Run #11 was performed in an attempt to measure the effect of placing a .001 inch separator between the plates. The new crop of water stored spores was used. Due to the unexpectedly low D-values of Run #10, Run #11 was only partially processed. The controls show a very high variability and therefore the remainder of the run was not processed. The treatment environment was dry bulb, 74°F, wet bulb, 57°F.

Run #12, October 24, 1968

The run attempted to compare the standard package with a package having a .0005 inch separator. The treatment was 65 minutes and the suspension of new, water-stored spores was used. The counts are too low for a good comparison between the standard package and the package with the separator. The grand mean of the standard packages is 22 and that of the packages with separators is 9.4; however, the data is highly variable.

Run #13, October 28, 1968

The run was a comparison between type

301 (smooth) and type 302 (less smooth) stainless steel mated surfaces. Treatments were wrapped control, 20 minutes, 40 minutes and 60 minutes. The spores were conditioned in an environment with 75°F dry bulb temperature and 55.5°F wet bulb temperature. They were treated in an environment with a 76°F dry bulb temperature and a 55.5°F wet bulb temperature. The test spores were from the new crop which has been stored in water and not frozen. The counts at 40 and 60 minutes on type 302 stainless steel are too low for great accuracy, as is the count at 60 minutes on type 301 stainless steel. However, it is clear that there is a significant difference between type 301 and 302 stainless steel surfaces in this particular experiment. We attribute the difference to the difference in surface finishes of the two materials and not to any difference in chemical composition of the steels. Other workers have shown that a variation between, for example, glass and stainless steel surfaces in open systems produces a very small variation in D-value.

The 60 minute package with type 302 stainless steel showed a recovery of zero spores; this is further confirmation of the extremely low contamination rate of the test and recovery system. Because of the variability problems the use of the new water stored spores will be discontinued and the alcohol stored spores will be used.

$D_{301\ (smooth)} = 18$ min.

$D_{302\ (less\ smooth)} = 9$ min.

Run #14, November 4-6, 1968

The run was used to measure the effect of delays between the preparation and packaging of the mated surface packages and the heating of the packages. It was necessary to measure the effect since a marathon run will be carried out to measure more exactly the effect of treatment dew point; delays between packaging and heating of the packages will be required in the experimental design.

A 65 minute treatment after the normal conditioning time gave a total recovery of

992 spores from two baseplates. After a 24 hour delay the recovery was 228 spores. After a 48 hour delay the recovery was 384 spores. Because of these results the starting time of the marathon run will be delayed for about 24 hours past the normal conditioning time of the spores used in the first 13 runs.

The old spores with a history of alcohol storage were used in this run and will be used in future runs until the cause of variability in the new water stored spore crop is pinpointed and cured.

The dew point of the conditioning environment was approximately 55°F.

$D_{\text{normal conditioning}}$ = 33 min.

$D_{24 \text{ hour delay}}$ = 25 min.

$D_{48 \text{ hour delay}}$ = 26 min.

D_{mean} = 28 min.

CONCLUSIONS

The conditioning and treatment humidities of the test system have a major effect on the D-value which is measured. Whether the conditioning or the treatment humidity is more important cannot be determined from present data. Whether humidity should refer to relative humidity, dew point or the partial pressure of water vapor in the system cannot be determined at this time. It is likely that during conditioning the relative humidity is the parameter which determines the amount of water in the spore; it would be in keeping with the behavior of hygroscopic materials whose water equilibrium is determined largely by the relative humidity. During treatment the relative humidity is extremely low and is not completely measurable since the treatment relative

humidity depends on the relative humidity of the environment and on the amount of water released from the test system; the spore alters the humidity of its microenvironment by releasing water.

Even prior to further analysis there is definitely a relation between survival and location of the spore deposit on the base plate. Row A at the open end of the package shows a lower survival than do the more protected rows. There is apparently some diffusion out of the sides and rear of the mated surface system since position B2 at the center of the mated surface usually shows the best survival; it is not unexpected since the package does not give a tight seal to the edge of the mated surface. The elevated recovery at position B2 also indicates that a larger mated surface would give a still higher recovery in the center. Therefore, the D-values which are quoted are by no means the highest which may be obtained in a mated surface system.

Surface finish has an effect on D-value with the rougher surface giving a lower survival; the rougher surface which was used is brushed in the direction of maximum diffusion which probably accentuates the effect. It is likely that a rougher surface will give still lower D-values by providing more pathways for diffusion; whether a more polished surface will give significantly higher D-values is unknown although at least a slight rise seems likely.

The effect of placing spacers between the mated surfaces is not clear at this point; however, we can say that thick spacers should give results comparable to an open system.

The handling history of the spores has a clearcut effect on their survivability and on their reliability of behavior. The older crop of spores used in most of the experimental run had been stored in alcohol suspension in a freezer for about one year. It showed longer D-values and higher reliability than a new crop of spores which had been stored in water and had not been frozen.

FUTURE WORK

The most important effects to be measured are the effects of conditioning and of treatment humidities. To measure the effects, a series of experiments has been designed based on the dew point of the conditioning and treatment systems. Three conditioning dew points (38°F, 55°F and 70°F) and four treatment dew points (38°F, 55°F, 63°F and 70°F) will be tested; the dew points have been chosen because they are adequately separated and are conveniently available in the laboratory. An extra treatment dew point has been inserted since it represents the upper level of dew points which might appear in a working environment. The experiment will be carried out in three sections. A batch of base plates will be made up and conditioned at one humidity; it will be

subdivided and treated in each of the four treatment environments. Four replicates will be treated at each of three time periods in each of the four treatment environments. For each conditioning environment, 48 mated surface packages will be treated. The first section of the experiment, using a conditioning dew point of 55°F, has been carried out but the results will not be processed in time for inclusion in this report. The raw data fall in line with the conclusions above.

Two questions may be attacked by placing separators of varying thickness between the mated surface. We should know the separator thickness required to make the mated surface system approach the behavior of an open system. Secondly, the use of a channeled separator will make diffusion insignificant except along the channel thereby removing the effect of diffusion out of the side and rear of the mated surface.

The z-value of the system will be measured to see if it compares with the results obtained in other systems. A low temperature in a mated surface system may have a unique effect; since the behavior of the system appears to depend on diffusion, by allowing a much longer time for diffusion, a low temperature treatment might tend to give a lower survival than would be predicted on the basis of temperature alone. Whether the effect can be detected in the present

system is not known.

The use of gasketing materials between the mated surfaces has been contemplated. They could be used to seal the sides and rear of the mated surface, thereby simplifying the diffusion pattern or they could be used as an interleaf to see if they would decrease the D-values; a decrease in mated surface D-values might help to decrease the sterilization time for spacecraft.

It would be possible to study a larger mated surface system; however, it would require construction of a new heat block unit and the cost per run would be greatly increased since the mated surfaces would have to be specially fabricated. Therefore, such a test is not contemplated at present.

ACKNOWLEDGEMENTS

The technical assistance of Phyllis Streeter, Ellen Kukla and Julie Haugen in the microbiological aspects of the project is gratefully acknowledged.

We also acknowledge David Lang's assistance with the calibration and improvement of the heat block device.

EXAMPLES OF RAW DATA

The semi raw-data sheets of three typical experimental runs illustrate the general characteristics of the data which are being collected. Duplicate plate counts and dilution factors have been processed into estimates of numbers of spores for clarity.

RUN NO. 6 DATE OF HEATING 9-25-68

Treatment
Time (Min.) 0 ;Temp. — °C;Package Wrap Control-Reg
9 Data Points, 2 Subdata per Data Point

$\bar{x}$ = 259 Pl. Cts. (1.30×10^5 Spores)
s_{data} = 20.1 (7.8 %)

Data Map: No. of Spores (All numbers x 10^5)
Row A at open end of package

Row \ Column	1	2	3	Row. Av.
C	~~1.35~~	1.29	1.41	
B	1.19	1.39	1.38	
A	1.11	1.25	1.32	
Col. Av.				

Remarks:

Treatment
Time (Min.) 0 ;Temp. — °C; Package Wrap Control-Wet
9 Data Points, 2 Subdata per Data Point

$\bar{x}$ = 266 Pl. Cts. (1.33×10^5 Spores)
s_{data} = 21.3 (8.0 %)

Data Map: No. of Spores (All numbers x 10^5)
Row A at open end of package

Row \ Column	1	2	3	Row Av.
C	1.15	1.30	1.28	
B	1.28	1.26	1.46	
A	1.38	1.42	1.42	
Col Av.				

Remarks:

Treatment
Time (Min.) 20 ;Temp. 125.8 °C;Package Reg
9 Data Points, 2 Subdata per Data Point

$\bar{x}$ = 56.4 Pl. Cts. (2.82×10^4 Spores)
s_{data} = 22 (39 %)

Data Map: No. of Spores (All numbers x 10^4)
Row A at open end of package

Row \ Column	1	2	3	Row. Av.
C	2.75	3.98	4.73	
B	2.15	2.90	3.63	
A	1.93	1.83	1.50	
Col. Av.				

Remarks:

RUN NO. 6 DATE OF HEATING 9-25-68

Treatment
Time (Min.) 40 ;Temp. 125.8 °C;Package Reg
9 Data Points, 2 Subdata per Data Point

$\bar{x}$ = 67.2 Pl. Cts. (3.36×10^4 Spores)
s_{data} = 26.2 (39 %)

Data Map: No. of Spores (All numbers x 10^4)
Row A at open end of package

Row	Column 1	2	3	Row. Av.
C	4.33	4.28	5.13	
B	3.38	4.50	3.25	
A	2.03	1.65	1.70	
Col. Av.				

Remarks:

Treatment
Time (Min.) 40 ;Temp. 125.8 °C; Package Wet
9 Data Points, 2 Subdata per Data Point

$\bar{x}$ = 178.5 Pl. Cts. (8.93×10^3 Spores)
s_{data} = 70.5 (39 %)

Data Map: No. of Spores (All numbers x 10^3)
Row A at open end of package

Row	Column 1	2	3	Row Av.
C	12.5	11.5	10.4	
B	10.5	12.1	10.1	
A	4.18	5.58	3.45	
Col Av.				

Remarks:

Treatment
Time (Min.) 80 ;Temp. 125.8 °C;Package Reg
9 Data Points, 2 Subdata per Data Point

$\bar{x}$ = 223 Pl. Cts. (1.12×10^3 Spores)
s_{data} = 150 (67 %)

Data Map: No. of Spores (All numbers x 10^2)
Row A at open end of package

Row	Column 1	2	3	Row. Av.
C	11.8	2.60	16.7	
B	9.43	16.4	8.40	
A	5.45	1.93	4.55	
Col. Av.				

Remarks:

Treatment
Time (Min.) 160 ;Temp. 125.8 °C;Package Reg
9 Data Points, 2 Subdata per Data Point

$\bar{x}$ = 24 Pl. Cts. (120 Spores)
s_{data} = 14 (57 %)

Data Map: No. of Spores (All numbers x 1)
Row A at open end of package

Row	Column 1	2	3	Row. Av.
C	170	195	50	
B	85	143	225	
A	60	30	123	
Col. Av.				

Remarks:

RUN NO. 6 DATE OF HEATING 9-25-68

Treatment
Time (Min.) 160 ;Temp. 125.8 °C; Package Wet
9 Data Points, 2 Subdata per Data Point
$\bar{x}$ = [redacted] Pl. Cts. (104 Spores)
s_{data} = 11 (53 %)

Data Map: No. of Spores (All numbers x 1)
Row A at open end of package

Row \ Column	1	2	3	Row Av.
C	133	43	45	
B	198	143	38	
A	125	130	80	
Col Av.				

Remarks:

RUN NO. 8 DATE OF HEATING 10-3-68

Treatment
Time (Min.) 0 ;Temp. 125.9 °C;Package wrap control
9 Data Points, 2 Subdata per Data Point
$\bar{x}$ = [redacted] Pl. Cts. (1.38×10^5 Spores)
s_{data} = .05 [redacted] (3.6 %)

Data Map: No. of Spores (All numbers x 10^5)
Row A at open end of package

Row \ Column	1	2	3	Row. Av.
C	1.33	1.34	1.38	
B	1.41	1.41	1.31	
A	1.45	1.42	1.33	
Col. Av.				

Remarks:

Treatment
Time (Min.) 20 ;Temp. 125.9 °C;Package U
9 Data Points, 2 Subdata per Data Point
$\bar{x}$ = Pl. Cts. (3.46×10^4 Spores)
s_{data} = 1.22 (35 %)

Data Map: No. of Spores (All numbers x 10^4)
Row A at open end of package

Row \ Column	1	2	3	Row. Av.
C	4.40	4.03	4.65	
B	3.98	3.98	4.48	
A	1.53	1.78	2.35	
Col. Av.				

Remarks:

Treatment
Time (Min.) 20 ;Temp. 125.9 °C; Package S
9 Data Points, 2 Subdata per Data Point
$\bar{x}$ = Pl. Cts. (3.22×10^4 Spores)
s_{data} = .55 (17 %)

Data Map: No. of Spores (All numbers x 10^4)
Row A at open end of package

Row \ Column	1	2	3	Row Av.
C	2.33	3.45	3.03	
B	4.18	2.98	3.23	
A	2.83	3.08	3.23	
Col Av.				

Remarks:

RUN NO. 8 DATE OF HEATING 10-3-68

Treatment
Time (Min.) 50 ;Temp. 125.9 °C;Package U
9 Data Points, 2 Subdata per Data Point

$\bar{x}$ = _____ Pl. Cts. (9.76×10^3 Spores)
s_{data} = 7.8 (80 %)

Data Map: No. of Spores (All numbers x 10^3)
Row A at open end of package

Row \ Column	1	2	3	Row. Av.
C	1.07	19.5	22.6	
B	13.3	12.7	9.03	
A	2.90	2.93	3.80	
Col. Av.				

Remarks:

Treatment
Time (Min.) _____ ;Temp. 125.9 °C; Package S
9 Data Points, 2 Subdata per Data Point

$\bar{x}$ = _____ Pl. Cts. (9.82×10^3 Spores)
s_{data} = 2.3 (23 %)

Data Map: No. of Spores (All numbers x 10^3)
Row A at open end of package

Row \ Column	1	2	3	Row Av.
C	11.3	9.53	8.25	
B	13.8	12.4	7.65	
A	7.00	9.53	8.93	
Col Av.				

Remarks:

Treatment
Time (Min.) 110 ;Temp. 125.9 °C;Package U-301
9 Data Points, 2 Subdata per Data Point

$\bar{x}$ = _____ Pl. Cts. (10.6×10^3 Spores)
s_{data} = 8.7 (83 %)

Data Map: No. of Spores (All numbers x 10^3)
Row A at open end of package

Row \ Column	1	2	3	Row. Av.
C	20.7	20.0	21.2	
B	11.0	10.6	10.0	
A	.910	.268	.330	
Col. Av.				

Remarks: Row C estimated from $\frac{1}{2}$-plate counts

Treatment
Time (Min.) _____ ;Temp. 125.9 °C; Package S-301
9 Data Points, 2 Subdata per Data Point

$\bar{x}$ = _____ Pl. Cts. (5.80×10^3 Spores)
s_{data} = 4.0 (68 %)

Data Map: No. of Spores (All numbers x 10^3)
Row A at open end of package

Row \ Column	1	2	3	Row Av.
C	8.13	11.2	9.20	
B	5.80	8.80	6.18	
A	1.13	1.13	.595	
Col Av.				

Remarks:

RUN NO. 8 DATE OF HEATING 10-3-68

Treatment
Time (Min.) 110 ;Temp. 125.9 °C;Package U-302
9 Data Points, 2 Subdata per Data Point

$\bar{x}$ = _____ Pl. Cts. (3.42×10^3 Spores)
s_{data} = 3.8 (112. %)

Data Map: No. of Spores (All numbers x 10^3)
Row A at open end of package

Row \ Column	1	2	3	Row. Av.
C	8.20	9.18	7.93	
B	1.42	1.93	1.67	
A	.128 [redacted]	.070	.258	
Col. Av.				

Remarks:

RUN NO. 13 DATE OF HEATING 10-28-68

Treatment
Time (Min.) 0 ;Temp. — °C;Package Wrap Control-301
9 Data Points, 2 Subdata per Data Point

$\bar{x}$ = _____ Pl. Cts. (1.56×10^5 Spores)
s_{data} = .29 (19 %)

Data Map: No. of Spores (All numbers x 10^5)
Row A at open end of package

Row \ Column	1	2	3	Row. Av.
C	1.22	1.68	1.80	
B	1.63	1.67	1.60	
A	1.83	.933	1.70	
Col. Av.				

Remarks:

Treatment
Time (Min.) 0 ;Temp. — °C; Package Wrap Control-302
9 Data Points, 2 Subdata per Data Point

$\bar{x}$ = _____ Pl. Cts. (1.46×10^5 Spores)
s_{data} = .29 (20 %)

Data Map: No. of Spores (All numbers x 10^5)
Row A at open end of package

Row \ Column	1	2	3	Row Av.
C	1.67	1.61	~~0~~	
B	1.57	1.61	1.10	
A	.945	1.44	1.76	
Col Av.				

Remarks:

Treatment
Time (Min.) 20 ;Temp. 125.2 °C;Package 301
9 Data Points, 2 Subdata per Data Point

$\bar{x}$ = _____ Pl. Cts. (4.22×10^3 Spores)
s_{data} = 3.5 (83 %)

Data Map: No. of Spores (All numbers x 10^3)
Row A at open end of package

Row \ Column	1	2	3	Row. Av.
C	3.25	2.68	4.58	
B	6.85	6.03	11.4	
A	* .15	2.58	* .43	
Col. Av.				

Remarks: * plate ct. too low for accuracy

RUN NO. 13 DATE OF HEATING 10-28-68

Treatment
Time (Min.) 20 ;Temp. 125.2 °C; Package 302
9 Data Points, 2 Subdata per Data Point
$\bar{x}$ = _____ Pl. Cts. (4.8×10^2 Spores)
s_{data} = 3.4 (69 %)

Data Map: No. of Spores (All numbers x 10^2)
Row A at open end of package

Row \ Column	1	2	3	Row Av.
C	0	1.0	3.3	
B	3.5	9.8	5.5	
A	5.0	9.5	6.0	
Col Av.				

Remarks:
Plate Counts too low for accuracy.

RUN NO. 13 DATE OF HEATING 10-28-68

Treatment
Time (Min.) 40 ;Temp. 125.2 °C;Package 301
9 Data Points, 2 Subdata per Data Point
$\bar{x}$ = _____ Pl. Cts. (10.6×10^2 Spores)
s_{data} = 9.5 (90 %)

Data Map: No. of Spores (All numbers x 10^2)
Row A at open end of package

Row \ Column	1	2	3	Row. Av.
C	.75	1.38	.78	
B	16.4	10.4	6.23	
A	9.82	24.5	24.8	
Col. Av.				

Remarks:

Treatment
Time (Min.) 40 ;Temp. 125.2 °C; Package 302
9 Data Points, 2 Subdata per Data Point
$\bar{x}$ = _____ Pl. Cts. (1.8 Spores)
s_{data} = _____ (%)

Data Map: No. of Spores (All numbers x 1)
Row A at open end of package

Row \ Column	1	2	3	Row Av.
C	0	0	0	
B	0	3	0	
A	8	0	5	
Col Av.				

Remarks:

Treatment
Time (Min.) 60 ;Temp. 125.2 °C;Package 301
9 Data Points, 2 Subdata per Data Point
$\bar{x}$ = _____ Pl. Cts. (32 Spores)
s_{data} = 49 (151 %)

Data Map: No. of Spores (All numbers x 1)
Row A at open end of package

Row \ Column	1	2	3	Row. Av.
C	3	8	3	
B	138	93	20	
A	10	3	13	
Col. Av.				

Remarks:

RUN NO. 13 DATE OF HEATING 10-28-68

Treatment
Time (Min.) 60 ;Temp. 125.2 °C; Package 302

9 Data Points, 2 Subdata per Data Point

$\bar{x}$ = _____ Pl. Cts. (0 Spores)

s_{data} = _____ (_____ %)

Data Map: No. of Spores (All numbers x 1)
Row A at open end of package

Row \ Column	1	2	3	Row Av.
C	0	0	0	
B	0	0	0	
A	0	0	0	
Col Av.				

Remarks:

DETECTION OF LOW LEVELS OF MICROBIAL CONTAMINATION ON SURFACES BY CHEMICAL APPROACHES

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INTRODUCTION

In the previous Progress Report, covering the period December 1, 1967, through May 31, 1968, a chemical method was described with which it was possible to detect as few as 50 microbial cells on a solid surface in 45 minutes. It may be possible to reduce the number of cells examined by a factor of 10; however, this will require additional instrumentation. This equipment is now on order.

The first step was to answer the question "How many bacteria are on a surface?" To answer this question chemically a quantitative analysis of some chemical known to be present in the cell is made. The second question is "Are the cells dead or alive?". This involves the study of the basic mechanism that produces death, in terms of chemical reactions and their end products. Here the assumption is that the death of the cell is caused by a non-reversible chemical reaction.

During the current period work has been initiated to answer this second question. In these initial experiments DNA and other nucleotides have been examined using chromatographic techniques to determine if heating of microbial

cells produced measurable chemical changes.

OBJECTIVE

The objective is to be able to use chemical methods to (1) determine the number of microbial cells present on a surface and (2) determine the relative percentages of living and dead cells.

EXPERIMENTAL PROCEDURE

In studies designed to differentiate between dead and live microorganisms, the separation of chemical compounds has been accomplished by using thin layer chromatography accompanied by infrared spectrophotometry for identification of compounds. The quantities of materials that are being analyzed are kept on a micro level; however, quantities are large enough so that they are nct influenced by contamination that may be present in the air since present work is being conducted at the bench top.

In these initial studies *E. coli* grown on agar plates in a standardized method are being used as the microbial substrate. At a later date *Bacillus subtilis* var. *niger* spores as well as other microbial vegetative cells and spores will be evaluated. Chromatographic plates were prepared ahead of time using purified cellulose powder and distilled water. After drying the plates, they were cleaned chromatographically in order to exclude any contaminants from the

air. A loop of E. coli from the agar plates was transferred to a test tube containing 0.5 ml of distilled water. Three microliters of this solution were applied to the previously prepared thin layer chromatography plates. Five sets of chromatography plates were prepared. The plates were transferred to a drying oven at 130°C for 30 minutes, one hour, three hours and 15 hours respectively. Following heat treatment, the heated plates, as well as the non-heated controls, were developed in the chromatographic chamber using a n-butanol acetone solvent system. After developing, the separated compounds were made visible in ultraviolet light and by heating. At this point the chromatograms were ready for evaluation by comparison with standard compounds used in the procedure and structural analysis in the infrared spectrophotometer. Some chemical changes have been observed.

RESULTS AND DISCUSSION

A definite increase in the separating patterns was established between the unheated material and the microorganisms heated for 15 hours at 130°C. It is possible that a separation was also produced for some of the shorter heating times; however this separation may have been caused by many factors and will be investigated further.

Preliminary tests have suggested that a great deal more work must be done to perfect the separation method by

exploring different organic solvent systems. At the same time, several different species of microorganisms using both vegetative cells and spores at different phases in the microbial growth cycle will have to be studied. It is possible that the separation pattern will be affected by different growth temperatures, different growth nutrients, age and other factors. The catalytic activity of enzymes will be determined in the heat affected material of each phase in order to determine the ability of the microorganisms to recover.

CONCLUSION

The results of the initial experiments suggest that it may be possible to observe chemical changes resulting from heating microorganisms.

FUTURE WORK

During the next period we plan to proceed to carry on the studies for reducing the quantity of organisms that can be identified and at the same time to proceed with this study of differentiating between live and dead bacteria by study of the separation patterns using different solvent systems and different microorganisms.

PUBLICATIONS AND PRESENTATIONS

DURING THIS REPORTING PERIOD

Anticipated Publication

1. Pflug, I. J.; "Sterilization Methods". A Chapter of "Planetary Quarantine: Principles, Methods and Problems" edited by Lawrence B. Hall. To be published as Monograph Number 6 in The Foundations of Space Biology and Medicine